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Liver X Receptors and Liver Physiology

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Abstract

The liver x receptors LXRα (NR1H3) and LXRβ (NR1H2) are members of the nuclear hormone receptor superfamily of ligand dependent transcription factors that regulate transcription in response to the direct binding of cholesterol derivatives. Studies using genetic knockouts and synthetic ligands have defined the LXRs as important modulators of lipid homeostasis throughout the body. This review focuses on the control of cholesterol and fatty acid metabolism by LXRs in the liver and how modifying LXR activity can influence the pathology of liver diseases.

Keywords

LXR; nuclear receptors; cholesterol; fatty acids; liver; NASH

1. Introduction

The liver x receptors LXRα (NR1H3) and LXRβ (NR1H2) are members of the nuclear hormone receptor superfamily of ligand regulated transcription factors. The original identification of both LXR subtypes was based upon DNA sequence homology to other superfamily members with no knowledge of their ligands [1, 2]. Clones encoding LXRα were isolated from a liver cDNA library and the mRNA was found to be highly expressed in the liver, hence the name [2]. Although the liver is a critical site of LXR activity, and the focus of this review, the name liver x receptor is somewhat of a misnomer. In humans and mice LXRα is expressed at relatively high levels in the liver, intestine, adipose, muscle, spleen, lung, adrenal gland, kidney, and in myeloid cells of the immune system. LXRβ is ubiquitously expressed. The two LXR subtypes are encoded by separate genes and the protein sequences are 61% identical/80 % similar when human sequences are compared. The largest differences between subtypes are found in the unstructured amino terminal regions while the DNA binding domains are highly conserved (77% identical/92% similar). The human and mouse LXRα proteins are 92% identical while the identity between human and mouse LXRβ is 85%.

Transient transfection assays and *in vitro* ligand binding experiments demonstrated that a subset of oxidized cholesterol derivatives (oxysterols) including 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 24(S), 25-epoxycholesterol, 25-hydroxycholesterol and 27-

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hydroxycholeserol bind directly to LXRs and can function as agonists [3–6]. Cholesterol precursors such as FF-MAS (14-demethyl-14-dehydrolanosterol), zymosterol, and desmosterol have also been reported as LXR agonists [4, 5, 7, 8]. Pharmacological approaches that inhibit cholesterol synthesis and block sterol uptake in cultured cells support a role from cholesterol derivatives as LXR ligands [9, 10]. Furthermore, genetic knockouts that deplete oxysterols or that increase the levels of cholesterol precursors are consistent with these molecules functioning as bona fide LXR ligands *in vivo* [11, 12]. Nevertheless, if individual cholesterol derivatives function as LXR ligands in specific tissues or during unique physiological or pathological responses remains an unanswered question in the field. In the liver cholesterol derived LXR ligands are likely to be continuously present and it is not known if ligand access to receptors is regulated or if liver LXRs ever exist in a non-liganded state. Future studies that disrupt the ability of LXRs to bind cholesterol-derived ligands could be an approach used to define ligand-dependent and ligand-independent LXR activities.

2. DNA Binding

As described in the introduction, the DNA binding domains of LXRα and LXRβ are highly conserved and both bind to DNA as heterodimers with retinoid x receptors (RXRs) serving as the dimeric partner [2]. *In vitro* experiments indicate that LXR-RXR heterodimers prefer to bind to direct repeats of the nuclear hormone receptor half site (AGGTCA) separated by 4 nucleotides (DR4) or inverted repeats separated by 1 nucleotide (IR1) [2, 13]. The binding site preferences determined *in vitro* have been largely confirmed by chromatin immunoprecipitation sequencing (ChIP-Seq) experiments using cells and in mouse liver tissue [7, 14–16]. Interestingly, treatment of mice with the potent synthetic agonist T0901317 results in significant increases in genome-wide LXR binding in the liver. T0901317 not only increases LXR binding to pre-existing sites detected in untreated livers but also promotes binding to more than 11,000 new locations [14]. The functional activity of most of the agonist-dependent binding sites, however, has not been determined. Similarly, the DNA sequence features that distinguish agonist-dependent from agonist-independent binding sites are not defined. For many nuclear receptors treatment with agonists increases dimerization with RXR [17, 18]. LXRα, the major LXR subtype in the liver, is also a relatively unstable protein that is rapidly degraded by ubiquitin-dependent proteolysis. Treatment with strong agonists, however, increase the half-life of LXRα [9]. Therefore, increases in dimerization and in the amount of LXR protein may contribute to the increased genome-wide DNA binding observed in livers of agonist treated mice. Synthetic agonists like T0901317 are often an order of magnitude more potent and significantly more efficacious than endogenous cholesterol derived LXR ligands [19]. Currently it is not known if endogenous ligands contribute to genome-wide LXR binding. Future studies using tissues depleted of endogenous ligands or expressing LXR mutants that cannot bind these molecules will be needed to determine if genome-wide binding can be regulated by endogenous ligands in physiological settings.

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3. LXR and Cholesterol Sensing

Cholesterol modulates membrane fluidity, functions as a signaling molecule and serves as a precursor for steroid hormones and bile acids. Excess intracellular cholesterol, however, is toxic necessitating tight regulation of uptake, excretion, and synthesis. The liver is the main location for regulating whole body cholesterol homeostasis and LXRα is the predominant LXR subtype expressed in this tissue. Analysis of individual genetic knockouts of each subtype indicates that the liver-specific LXR functions described in this review are largely LXRα dependent [20–22]. By regulating transcription in response to the direct binding of cholesterol derivatives that track intracellular cholesterol levels, LXRs function in a positively acting feed-forward pathway that promotes cholesterol excretion and catabolism (Figure 1). The details of the individual LXR-dependent pathways that contribute to feed-forward control will be described in later sections of this review. Cholesterol biosynthesis, on the other hand, is controlled by a classic negative feedback mechanism (Figure 1) [23, 24]. Expression of genes encoding enzymes required for cholesterol synthesis is controlled at the level of transcription by the sterol regulatory element binding protein 2 (SREBP2) transcription factor. SREBP2 is produced as a membrane bound inactive precursor imbedded in the endoplasmic reticulum (ER). Upon translocation to the Golgi, proteolytic processing releases mature SREBP2 that migrates to the nucleus and activates transcription. High levels of cholesterol in the ER inhibits the movement of SREBP2 to the Golgi leading to decreased expression of cholesterol synthesis enzymes [23, 24]. Interestingly, both the LXR and SREBP2 pathways converge at the low density lipoprotein receptor (LDLR) which functions as the major mediator for cholesterol uptake in the liver (Figure 1). SREBP2 controls expression of the LDLR gene [23, 24] while activation of LXR increases transcription of the inducible degrader of LDLR (IDOL). IDOL, also known as myosin regulatory light chain interacting protein (MYLIP), is an E3 ubiquitin ligase that targets LDLR for degradation [25, 26].

4. Cholesterol Transport and HDL

The process of transferring cholesterol from peripheral cells to high-density lipoprotein particles (HDL) followed by transport to the liver for excretion is known as reverse cholesterol transport (RCT; Figure 2) [27–29]. In peripheral cells LXRs regulate expression of the genes encoding the ATP-binding cassette transporters ABCA1 and ABCG1 that transfer intracellular cholesterol to HDL [30, 31], the first step in the RCT pathway (Figure 2). ABCA1 and ABCG1 are expressed in many tissues and can be induced by LXR agonists in these locations [30, 32–35]. Nevertheless, most studies examining the ability of LXRs to regulate cholesterol efflux to HDL have been carried out in macrophages using either primary cells *ex* vivo or established monocyte/macrophage-derived cell lines. By phagocytosing damaged and dying cells macrophages are often exposed to acute changes in cholesterol levels. Human genetics also suggests an important role for macrophage cholesterol efflux to HDL. Patients with null mutations in the gene encoding ABCA1 have a genetic syndrome named Tangier Disease and often present with accumulation of lipid loaded macrophages in lymph tissues [36]. Importantly, treatment of macrophages in culture with LXR agonists increases efflux of intracellular cholesterol to HDL [37–40]. Conversely, combined genetic knockout of ABCA1 and ABCG1 significantly decreases

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macrophage cholesterol efflux [41]. Cholesterol accumulation by macrophages in blood vessel walls is an essential step in the pathogenesis of atherosclerotic cardiovascular disease [29, 42]. The ability of LXR agonists to increase macrophage cholesterol efflux to HDL suggested potential roles for LXRs in limiting atherosclerosis by reducing the cholesterol burden within atherosclerotic plaque. Indeed, treatment with LXR agonists decreases or even promotes regression of atherosclerosis in animal models of cardiovascular disease while genetic knockout of LXRs increase disease burden [21, 43–46]. Even though LXR agonists stimulate macrophage cholesterol efflux *in vitro*, Breevoort et al. [37] demonstrated that macrophage LXR activity makes little or no contribution to the ability of LXR agonists to stimulate RCT *in vivo* using an assay that measures the movement of cholesterol from macrophages to liver and ultimately to the feces. In contrast, selective deletion of LXRα in the liver significantly impairs the ability of LXR agonists to increase the movement of macrophage derived cholesterol [37]. These studies suggest that activity of LXRα in the liver plays a major role in controlling the movement of cholesterol out of macrophages and perhaps from other organs and tissues throughout the body.

Liver LXR activity modulates RCT *in vivo* by at least 3 mechanisms (Figure 2). First, as described in the followings section, by regulating cholesterol excretion and catabolism LXRα controls the overall flux through the RCT pathway. Second, studies by Zhang et al. [46] and Breevoort et al. [37] indicate that LXRs modulate the ability of HDL particles to accept cholesterol from macrophages. The LXR-dependent regulation of HDL cholesterol acceptor activity correlates with changes in HDL phospholipid composition which are described in section 6 [37]. Finally, LXR regulates the overall number of HDL particles at least in part by controlling expression of ABCA1 in the liver and intestine [37, 47]. By transferring intracellular cholesterol to newly synthesized lipid-poor HDL particles ABCA1 is also necessary for the biogenesis of HDL [29, 48, 49]. The observation that Tangier Disease patients have little or no circulating HDL is also consistent with a role for ABCA1 in HDL biogenesis [36]. Tissue-specific knockouts of ABCA1 and LXRα indicate that LXR agonists primarily increase HDL levels by regulating ABCA1 expression in the intestine in mice maintained on normal chow diets [47]. Interestingly, when mice are placed on high cholesterol diets a role for liver LXRα in regulating HDL particle number becomes apparent [37]. How dietary cholesterol uncovers a role for liver LXR activity in controlling HDL remains to be determined. Despite strong evidence for LXR-dependent regulation of HDL cholesterol levels, HDL particle number and HDL function in mice, a single LXR agonist had no effect on HDL cholesterol levels after multiple dosing in humans [50]. HDL particle number and function were not reported. In humans LXRs regulate expression of the gene encoding the cholesterol ester transport protein (CETP) an enzyme that transfers cholesterol from HDL to LDL particles [51–53]. CETP is not expressed in rodents and this species-specific difference in lipoprotein remodeling may contribute to the failure to detect increases in HDL cholesterol in humans treated with LXR agonists [50]. Importantly, studies in humans have failed to show beneficial effects of agents that raise HDL cholesterol [54–56] making it unlikely that LXR agonists will be developed for this purpose.

Scavenger receptor-B1 (SR-B1) is the major cell surface receptor for HDL cholesterol, however, cholesterol uptake from HDL does not require clathrin-dependent uptake or lysosomal targeting [57]. In the plasma membrane cholesterol can exist in inaccessible

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and accessible pools [58] and recently a family of endoplasmic reticulum (ER) anchored proteins, the Gram domain containing 1 proteins (GRAMD1A, 1B and 1C) also referred to as Aster proteins, have been shown to transfer accessible cholesterol from the plasma membrane to the ER [59, 60]. Cholesterol levels in the ER define the level of cholesterol synthesis by controlling the proteolytic processing of SREBP2 to an active transcription factor [23]. The Aster proteins therefore may function to link cholesterol arriving at the membrane from HDL to the regulation of cholesterol synthesis in the ER. In macrophages the gene encoding GRAMD1B was shown to be induced by LXR agonists and ChIP-Seq studies identified a binding site at this locus [60]. The regulation of GRAMD1B suggest that LXRs may act to facilitate the intracellular movement of cholesterol from the plasma membrane and provides a possible link between the feed-forward regulation of cholesterol transport/excretion by LXRs and the negative feedback control of cholesterol synthesis by SREBP2 (Figure 1). In the liver GRAMD1C, which is not regulated by LXR, appears to be the predominant GRAMD1 protein [60]. Wang et al., however, found that GRAMD1B is induced in the livers of mice fed a high cholesterol diet although the contribution of LXR to the diet-dependent regulation was not examined [61].

5. Cholesterol Excretion and Catabolism

Regulation of the ATP binding cassette transporters ABCG5 and ABCG8 in the liver plays a critical role in the ability of LXR agonists to stimulate biliary cholesterol excretion (Figure 2) [31, 62, 63]. ABCG5 and ABCG8 are half transporters that heterodimerize to form functional units [64]. Genetic knockouts and over expression studies indicate that ABCG5/G8 is required for the secretion of cholesterol into bile [62, 63, 65]. ABCG5/G8 also mediates the excretion of plant sterols from the intestine and mutations in the human ABCAG5/G8 genes leads to an inappropriate accumulation of plant sterols in the plasma referred to as sitosterolemia [64]. The genes encoding both subunits are expressed in opposite orientations from the same chromosomal locus and are controlled by a common bi-directional promoter in both mice and humans. Binding sites for LXR-RXR heterodimers have been functionally confirmed within the promoter and additional LXR binding sites throughout the locus have been identified by ChIP-Seq [14, 31]. Importantly, the ability of synthetic LXR agonists or high cholesterol diets to increase expression of ABCG5/G8 and enhance cholesterol excretion is lost in LXRα liver-specific knockout mice [46].

The catabolism of cholesterol to bile acids in the liver serves as an additional mechanism for controlling cholesterol levels. In rodents, LXRα directly regulates the classical pathway of bile acid synthesis by controlling expression of the gene encoding the rate limiting enzyme cholesterol 7α-hydroxylase (*Cyp7a1*; Figure 2) [46, 66]. As observed for ABCG5/G8, a well characterized LXR response element has been identified in the mouse and rat *Cyp7a1* promoters [66]. Treatment of mice with LXR agonists also decreases expression of the genes encoding 12α-hydroxylase (*Cyp8b1*) and oxysterol 7α-hydroxylase (*Cyp7b1*) [46, 66, 67]. 12α-hydroxylase sits at a branch point in the bile synthesis pathway and is necessary for the synthesis of cholic acid. The parallel arm in the pathway generates muricholic acid (Figure 3) [68]. Among bile acids tested, muricholic acid promotes the lowest amount of intestinal cholesterol absorption while cholic acid promotes the greatest amount [69, 70]. Oxysterol 7α-hydroxylase, on the other hand, functions in the alternative bile acid synthesis

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pathway to generate bile acids from oxysterols including those that function as endogenous LXR ligands (Figure 3) [68]. Combining LXR-dependent up-regulation of *Cyp7a1* with repression of *Cyp8b1* favors the catabolism of cholesterol to muricholic acid and limits the absorption of cholesterol in the intestine. Simultaneous repression of *Cyp7b1* maintains the hepatic pool of endogenous LXR ligands (Figure 3). The molecular mechanism(s) describing LXR agonist-dependent repression of *Cyp7b1* and *Cyp8b1* has not yet been determined. Importantly, there is little evidence for LXR-dependent regulation of bile acid synthesis in humans. The LXRE found in the mouse *Cyp7a1* promoter is not conserved in the human gene [71]. Humans also make chenodeoxycholic acid in place of muricholic acid [68] and chenodeoxycholic acid is better at promoting intestinal cholesterol absorption [69, 70].

Cholesterol and bile acid synthesis follow a circadian rhythm with peaks in the early part of the dark phase in rodents when feeding and activity begin to increase [72]. Nevertheless, little is known about how LXR activity interfaces with the circadian clock. Retinoid-related orphan receptor alpha (RORα) is an additional cholesterol regulated nuclear receptor that positively regulates expression of brain and muscle ARNT-like protein 1 (Bmal1) a core component of the circadian clock [73–75]. Studies by Wada et al. indicate that RORα and LXRs mutually suppress each other *in vivo* [76, 77]. LXRα has also been shown to positively regulate expression of the gene encoding basic helix-loop-helix family member e40 (BHLHE40, also known as DEC1) another transcription factor implicated in circadian control [78]. Studies examining circadian rhythms and lipid metabolism in liver-specific LXR knockout mice will be needed to understand how LXR activity interacts with the hepatic clock.

6. Fatty Acids, Triglycerides and Phospholipids

Along with regulating cholesterol transport, analysis of genetic knockouts and synthetic ligands identified critical roles for LXRs in the regulation of fatty acid and triglyceride synthesis [79, 80]. Early studies identified the gene encoding sterol regulatory element binding protein 1c (SREBP1c), itself a master transcriptional regulator of fatty acid synthesis, as a direct LXR target gene [19, 81]. Nevertheless, synthetic LXR agonists still increase fatty acid and triglyceride synthesis in SREBP1c knockout mice [82] suggesting, at least in response to strong pharmacological agonists, that LXRα can control fatty acid synthesis independent of SREBP1c. Subsequent studies have identified binding sites for LXRs in regulatory regions of genes encoding enzymes involved fatty acid synthesis, fatty acid chain elongation and fatty acid desaturation [66, 83–85]. LXR was also shown to regulate expression of the carbohydrate response element binding protein (ChREBP) a third lipogenic transcription factor [86–88]. It is likely that SREBP1c, ChREBP and LXRα function to coordinately regulate hepatic fatty acid synthesis in response to in-coming signals such as insulin, glucose and changing cholesterol levels.

Storing excess cholesterol as fatty acid-esters protects cells from the toxic effects of free cholesterol. This protective mechanism has been suggested as one reason for coupling cholesterol transport and fatty acid synthesis via LXR activity [80]. Treatment with synthetic LXR agonists, however, also increases the secretion of the triglyceride rich very low

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density lipoprotein particles (VLDL) leading to significant increases in plasma triglycerides [89–91]. In rodents which mostly carry plasma cholesterol in HDL particles, increasing VLDL secretion may be of little consequence. Indeed, LXR agonists strongly reduce atherosclerosis in mouse models of cardiovascular even in the face of large increases in plasma triglycerides [21, 43, 45, 92]. On the other hand, in non-human primates and in humans LXR agonists lead to increases in low density lipoprotein (LDL) cholesterol [25, 50, 51] that most likely arises from the remodeling of VLDL in the blood. The hyperlipidemic effects of synthetic LXR agonists have proved to be a large hurdle slowing the clinical development of these agents. Not surprisingly there have been large drug discovery efforts focused on identifying LXR agonists that maintain the beneficial effects on cholesterol transport while limiting hyperlipidemia. Unfortunately, while LXR agonists with improved therapeutic profiles in rodents and non-human primates have been identified the most advanced of these compounds failed to maintain separation of hyperlipidemia from cholesterol transport upon repeated dosing in humans [50]. In an elegant series of experiments using hydrogen-deuterium exchange Belorusova et al. [93] recently determined that LXR ligands which dissociate hyperlipidemia from cholesterol transport in animal models preferentially stabilize a region of the ligand binding domain (LBD) located in helix 3. These dissociated ligands, however, do not stabilize helix 12 also referred to as activation function 2 (AF2). Stabilization of helix 12 by packing on the surface of the LBD is thought to be the major mechanism of ligand-dependent activation of nuclear receptors [94]. The work of Belorusova suggests identifying ligands that stabilize helix 3 without impacting the dynamics of helix 12 may provide a rational approach for the identification of LXR ligands with improved therapeutic profiles *in vivo*.

LXRα directly regulates expression of several fatty acid elongases and desaturases in the liver and treatment with agonists leads to increases in long chain unsaturated fatty acids [83, 85, 95, 96]. LXRs are also critical regulators of the gene encoding lysophosphatidylcholine acyltransferase 3 (LPCAT3) an enzyme that mediates the incorporation of fatty acids at the *sn*2 position of phospholipids [97, 98]. Thus, activation of LXRs leads to remodeling of phospholipid composition by increasing the presence of long chain desaturated fatty acids particularly arachidonoyl containing phospholipids. Importantly, mice lacking LPCAT3 in the liver exhibit reduced plasma triglycerides, hepatic steatosis, and secrete lipid-poor VLDL lacking arachidonoyl phospholipids [99–101]. Mechanistic studies indicate that arachidonoyl phospholipids enhance the lipidation of VLDL particles as they are produced in the ER. LXRα is also responsible for hepatic expression of the gene encoding the phospholipid transport protein (PLTP) [102, 103], a second enzymes that plays a role in the lipidation and secretion of VLDL [104–108]. Thus, along with promoting the excretion and catabolism of cholesterol, activation of LXRs also drives the production and secretion of VLDL providing an additional mechanism to move cholesterol out of the liver. Although not directly demonstrated, it is likely that regulating the expression of LPCAT3 and PLTP modulates the phospholipid composition of HDL and contributes to the ability of hepatic LXRα to control HDL cholesterol acceptor activity described in section 4 [37].

Increased levels of phospholipids with desaturated fatty acids in the ER enhances vesicular transport to the Golgi facilitating movement of unprocessed SREBP1c [109]. In the Golgi, proteolytic processing of SREBP1c releases the active transcription factor which migrates

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to nucleus and increases expression of genes involved in fatty acid synthesis [23]. Thus, LXRα regulates hepatic fat synthesis at multiple levels (Figure 4). First, LXRα directly regulates expression of genes encoding enzymes involved in fat synthesis. Second, LXRα directly regulates expression of SREBP1c; itself a transcription factor regulating fatty acid synthesis. Third, by altering phospholipid composition LXRα indirectly increases SREBP1c-dependent transcription by increasing ER to Golgi transport. Finally, LXRα facilitates the lipidation and secretion of triglyceride rich VLDL particles. LXR mediated changes in phospholipid composition have also been shown to reduce ER stress and inflammation which may function to protect the ER when cholesterol levels rise [98].

The ability of synthetic LXR ligands and high cholesterol diets to increase bile acid synthesis and biliary cholesterol excretion is lost in LXR knockout mice. Nevertheless, no difference in these parameters or in expression of the relevant genes are detected when normal chow fed LXR positive and LXR knockout mice are compared [46, 66]. In contrast, as described in section 6, hepatic fatty acid synthesis is significantly down-regulated in normal chow fed LXR knockout mice compared to controls [21, 22, 46, 66, 110]. These observations suggest that different LXR-dependent gene networks can differ in their modes of regulation. Fatty acid synthesis genes are sensitive to genetic deletion of LXRs under all conditions. Cholesterol excretion/catabolism genes only appear to require LXR to respond to high dietary cholesterol or to potent synthetic LXR ligands. We suggest that there is a subset of genes with relatively low affinity LXR binding sites (e.g., cholesterol excretion/catabolism) which are only significantly occupied by LXRs when relatively high concentrations of ligands are achieved (see section 2). Genes with high affinity binding sites (e.g., fatty acid synthesis), however, are regulated under both low and high ligand concentrations (Figure 5).

Hepatic fatty acid synthesis is reported to be elevated in patients with non-alcoholic fatty liver disease (NAFLD) [111, 112]. NAFLD is currently estimated to affect 25% of the adult population in the United States and is linked to the increasing world-wide obesity epidemic [111, 113]. Synthetic LXR antagonists decrease hepatic fatty acid synthesis and reduce hepatic triglyceride accumulation in animal models of NAFLD suggesting potential therapeutic roles for such molecules [114, 115]. Nevertheless, the etiology of NAFLD is complicated. Approximately 25% of NAFLD patients progress to non-alcoholic steatohepatitis (NASH). NASH is characterized by immune infiltration of the liver, large lipid filled hepatocytes (hepatocyte ballooning), and fibrosis. Importantly, NASH increases the risks for cirrhosis, hepatocellular carcinoma, and liver failure [113]. The processes triggering the progression of NAFLD to NASH or even if there is a true stepwise progression from one pathological state to the other remain to be determined. Elevated liver cholesterol is also observed in patients with NASH and non-esterified (free) cholesterol correlates with disease severity [116–119]. Furthermore, clinical studies indicate that patients with NASH can benefit from inhibiting cholesterol synthesis with statins [120–123]. High intracellular cholesterol is toxic leading to endoplasmic reticulum stress, inflammation, and cell death all of which have been suggested to contribute to NASH [113]. Studies implicating elevated hepatic cholesterol in NASH raise questions regarding the potential utility of inhibiting LXR activity with small molecule antagonists that may also increase hepatic cholesterol (see section 5). Consistent with these concerns are studies with LXR

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knockouts suggesting that decreasing LXR activity increases liver fibrosis [124, 125]. Thus, genetic deletion of LXRs and pharmacological inhibition of LXR activity paradoxically leads to opposite and conflicting results in liver disease models.

7. Carbohydrate Metabolism and Type II Diabetes

Type II diabetes is often associated with elevated hepatic fatty acid synthesis and increases in plasma triglyceride levels. Since synthetic LXR agonists can promote profound hypertriglyceridemia it was quite surprising that these same molecules were shown to have significant anti-diabetic activity in models of type II diabetes including *db/db* mice and high fat fed animals [126–129]. Several mechanisms and sites of action for the anti-diabetic activity of LXR agonists have been described. Importantly, Commerford et al. used euglycemic-hyperinsulinemic clamp measurements of high fat fed rats to conclude that inhibition of hepatic glucose production accounts for a large majority of LXR anti-diabetic activity [127]. Consistent with the results of Commerford et al., treatment with LXR agonists also decreases expression of phosphoenolpyruvate carboxykinase (PCK) and other hepatic gluconeogenic enzymes [126, 129]. ChIP-Seq studies have identified potential LXR binding sites in the promoter regions of PCK and glucose-6-phosphatase, however, it is not known if LXRs directly repress gluconeogenic gene expression or if the inhibitory effect of activating LXR is indirect [14]. Hypoxic inducible factor 1 alpha (HIF1α) is known to induce glycolysis under condition of low oxygen. Recent studies suggest that LXRs can induce the gene encoding HIF1α in macrophages [130, 131]. If an LXR-HIF1α pathway contributes to hepatic carbohydrate metabolism, however, has not been determined. By simultaneously promoting fatty acid synthesis and inhibiting hepatic glucose production, potent LXR agonists partially mimic the activity of insulin in the liver. Consistent with an “insulin-like” function, in adipose LXRs have been shown to regulate expression of the gene encoding GLUT4 [128, 129], the major insulin stimulated glucose transporter. Therefore, when viewed in the context of the established roles for LXRs in regulating hepatic cholesterol and fatty acid metabolism, the ability to coordinately regulate carbohydrate metabolism suggests a broader role for LXRs as integrators of metabolic signals that identify the fed-state. In that regard Mitro et al. provided a crystal structure indicating that glucose can directly bind to the LXR ligand binding pocket and suggested that LXRs may directly sense glucose levels [132]. There has, however, been little follow up on this study.

8. Kupffer Cells and Hepatic Stellate Cells

Kupffer cells are tissue resident macrophages of the liver and lineage tracing experiments identified LXRα as a transcription factor required to determine and to maintain Kupffer cell fate [133, 134]. The Kupffer cell fate-determining LXR gene network includes genes such as *Cdh5*, *Pcolce2*, *Kcna2*, and *Il18bp* and is distinct from the well characterized LXR-dependent networks controlling cholesterol and fatty acid metabolism that are also LXR regulated in these cells [134–137]. Kupffer cells are derived from the yolk sac and invade the embryo at the onset of organogenesis [133]. Following selective depletion of Kupffer cells in adult animals, however, bone marrow derived monocytes migrate to the perisinusoidal space of the liver where the combinatorial activation of the Notch and transforming growth factor

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beta (TGFβ) pathways leads to rapid induction of LXRα and the expression of Kupffer cell-specific genes in these cells [135, 136]. Similar Notch and TGFβ dependent pathways appear to act during normal Kupffer cell development [135]. Interestingly, exposure of mice to a high fat/high fructose/high cholesterol diet reported to promote NASH leads to decreased expression of Kupffer cell-specific genes in enriched populations of liver myeloid cells. Genome-wide analysis suggests decreased binding of LXRα at regulatory regions controlling Kupffer cell fate determining genes and relocalization to regions controlling genes associated with increased lipid burden and tissue scarring [137]. Therefore, as has been seen with other macrophage populations, Kupffer cells can assume distinct phenotypes in response to changing environmental signals. The main driver of this phenotypic switch appears to be a diet-dependent increase in the levels of activating transcription factor 3 (ATF3) which functions to drive LXRα to new regulatory regions [137]. Fibrosis and an inappropriate tissue scarring/wound healing response are associated with NASH raising the possibility that diet-dependent alterations in Kupffer cell LXR activity contribute to rising incidence of this disease.

Hepatic stellate cells are an additional cell type in the liver that plays critical roles in the pathology of liver disease [113, 138]. Stellate cells reside in the space between sinusoidal endothelial cells and the surface of hepatocytes referred to as the Space of Disse. In normal liver physiology these cells function as the major storage site for vitamin A [138]. Upon insult or injury stellate cells become activated, assume a myofibroblast phenotype and secrete numerous extracellular matrix proteins driving fibrosis. Importantly, the activation of stellate cells plays a critical role in the transformation of NAFLD to NASH [113, 138]. Signals from lipid filled and/or apoptotic hepatocytes as well as from pro-inflammatory immune cells including TGFβ, osteopontin, platelet derived growth factor, hedgehog ligands, chemokines and danger associated molecular patterns (DAMPS) all can activate stellate cells leading to increased extracellular matrix production and fibrosis [113]. Cholesterol accumulation has been shown to sensitize hepatic stellate cells to the pro-fibrotic action of TGFβ [139], raising the possibility that LXR dependent regulation of cholesterol metabolism may be important in this cell type. Consistent with a role for LXR in stellate cells, Beaven et al. [124] demonstrated that LXR agonists suppress markers of fibrosis in hepatic stellate cells *ex vivo* while stellate cells isolated from LXR knockout mice express higher levels of fibrotic genes. Increased liver fibrosis was also observed in LXR knockout mice compared to control mice when the carbon tetrachloride and methionine-choline deficient diet models of liver damage were examined [124]. If the ability of LXRs to limit fibrosis is dependent on promoting cholesterol efflux from hepatic stellate cells, however, has not been addressed.

A single base polymorphism in the gene encoding patatin-like phospholipase domain-containing protein 3 (PNPLA3) that changes isoleucine at position 148 to methionine is genetically associated with large spectrum of a liver diseases including NAFLD and NASH [140]. Nevertheless, the function of PNPLA3 and how it contributes to liver disease remains to be determined. Bruschi et al. found that LXR transcriptional activity is impaired in cells expressing the PNPLA3 methionine 148 variant and that these cells accumulate more cholesterol *in vitro* compared to cells expressing PNPLA3 isoleucine 148 [141]. How PNPLA3 influences LXR function remains an open question. The work of Bruschi et al.

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does, however, raises the important possibility that LXR activity in stellate cells contributes to the susceptibility to liver disease.

9. Post-Translational Modifications

Like many nuclear receptors LXRs are post-translationally modified. Interestingly, 3 post-translational modifications appear to act largely through a single lysine residue in LXRα. Li et al demonstrated that acetylation of lysine 434 (K434; K432 in the mouse sequence) in the LXRα LBD decreases transcriptional activity [142]. The responsible acetyltransferase has not been identified, however, Sirtuin 1 (SIRT1) was shown to deacetylate K434 in a ligand-dependent manner. Consistent with an activating role for deacetylation, LXR transcription activity was reduced in livers from SIRT1 knockout mice. Deacetylation of K434 stimulates ubiquitination of this amino acid resulting in a decrease in protein half-life [142]. Several studies have shown that ligand-dependent transcriptional regulation by nuclear receptors is coupled to ubiquitin mediated degradation [143, 144] suggesting a potential link between an acetylation/deacetylation cycle and LXR transcriptional activity. K434 along with K328 of LXRα along with K410 and K448 of LXRβ can also be modified by SUMOylation. Ghisletti et al. [145] and Lee et al. [146] have suggested that SUMOylation is necessary for LXR-dependent anti-inflammatory activity. SUMOylated LXRs directly repress pro-inflammatory gene expression by either blocking the removal of transcriptional repressors gene regulatory elements stimulated nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and/or by inhibiting the DNA binding activity of signal transducer and activated transcription 1 (STAT1) [145, 146]. Taken together, these studies highlight the potential that cross-talk among enzymatic systems mediating the acetylation, SUMOylation and ubiquitination modulates LXR activity. Despite the functional consequences of the LXR modifications described above, Ito et al. found that introducing LXRα with both K328 and K434 changed to arginine into immortalized bone marrow derived macrophages derived from LXR knockout mice completely rescued both the positive regulation of LXR target genes as well as the repression of pro-inflammatory gene expression [147]. Most of the analysis of LXR post-translational modification has been carried out in cell culture systems. The apparent contradictions among these studies emphasizes the importance of using CRIPR and other genome modification approaches to introduce mutations of interest into the endogenous LXR loci to facilitate experiments *in vivo*.

Serine 198 of LXRα (S198; S196 in the mouse sequence) has been shown to be phosphorylated in a ligand-dependent fashion [148, 149]. Casein kinase 2 and protein kinase A have been suggested to be the responsible enzymes [149, 150]. Mutation of the serine to alanine in mice (S196A) alters the transcriptional response to LXR ligands with a shift to more anti-inflammatory gene expression profile in immune cells [151]. When placed on a high fat/high cholesterol diet female S196A mice exhibit elevated *de novo* fatty acid synthesis and increased hepatic triglyceride accumulation. The expression of LXR target genes involved in fatty acid synthesis such as SREBP1c is also enhanced in S196A mice relative to controls. Although steatosis is increased in livers from female S196A mice, hepatic inflammation and fibrosis is surprisingly decreased indicating that the progression from fatty liver to NASH is impaired [152]. It is tempting to speculate that the increase in steatosis and decrease in inflammation/fibrosis observed in S196A mice reflects differences

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in the gene networks regulated by LXRα in hepatocytes, immune cells, and hepatic stellate cells respectively. Future studies employing cell type specific expression of S196A will be needed to test this hypothesis. Less well studied LXR post-translational modifications include poly ADP-ribosylation (inhibitory) [153] and O-linked β-N-acetylglucosamine (O-GlcNAc; stimulatory) [154]. Modification of LXRs by O-GlcNAc increases with rising glucose levels suggesting a mechanism that potentially allows LXRs to function as a glucose sensor [154] (see Section 7).

10. Future Directions

Over the last 10 years the contributions of the LXRs, particularly LXRα, to liver physiology have been well studied. Nevertheless, many important questions related to LXR activity and to the potential therapeutic benefits of LXR ligands are still unanswered. The ability of oxysterols and other sterols to bind directly to LXRs and to regulate LXR dependent transcription has been well established [4, 5, 7, 8, 11, 12]. If individual endogenous LXR ligands are uniquely required during different physiological settings or to activate specific subsets of LXR regulated genes, however, is not known. The use of CRISPR to generate tagged LXRs along with improvements in mass spectrometry suggest that it should be possible to identify endogenous ligands directly bound to LXRs *in vivo*. The classic model of nuclear receptor activity describes a non-liganded inactive state with receptors bound to transcriptional corepressors. Agonists promote conformational changes that decrease corepressor binding and promote interactions with coactivator proteins that increase transcription [94]. Since it is hard to imagine that the liver is ever “cholesterol free” one can question if hepatic LXRs ever experience a non-liganded state and if the classic model of nuclear receptor activity is even applicable in this context. Future studies exploring the activity of LXR mutants that do not bind endogenous ligands could be used to explore ligand independent LXR activities.

The combination of potent synthetic LXR ligands and genetic knockouts have uncovered roles for LXRs in the transcriptional regulation of cholesterol, fatty acid, and carbohydrate metabolism [80]. LXR is generally considered to a be a cholesterol sensor but it is not obvious why the liver would coordinately regulate cholesterol, fatty acid, and carbohydrate metabolism in response to changing cholesterol levels. Cholesterol is a precursor required for the synthesis of several molecules that function as autocrine, paracrine, and endocrine signals including steroid hormones and bile acids. Perhaps then it is more appropriate to consider one or more endogenous LXR ligands as a classical hormonal signal and not simply as a surrogate marker of intracellular cholesterol levels. In such a system LXRα may function not as a cholesterol-regulated transcription factor but as a sensor of the fed state that like insulin promotes energy storage and inhibits gluconeogenesis. As described above, identifying ligands bound to LXR in cells and in liver tissue could shed light onto how LXR activity is regulated during normal physiology and in pathological settings.

The therapeutic potential of LXR ligands for treating liver diseases remains an important and unanswered question. The hyperlipidemic response to LXR agonists has been a major hurdle to the development of ligands for treating chronic metabolic diseases such as atherosclerosis and type II diabetes [50]. Limiting activity in the liver also may be necessary if LXR ligands

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are going to prove useful for other indications where they are currently being explored such as Alzheimer's disease and cancer. In hepatocytes genes encoding proteins involved in fatty acid synthesis and cholesterol excretion respond differentially to genetic knockout of LXRs. Expression of fatty acid synthesis genes such as SREBP1c are strongly decreased in LXRα knockouts while cholesterol excretion genes such as ABCG5 and ABCG8 are not. LXRα, however, is necessary for both subsets of genes to induced by high cholesterol diets or by synthetic LXR agonists [31, 46, 66]. What accounts for the gene-selective response to LXRα knockout is not known? A better understanding of the molecular basis for this selectivity, however, may provide the opportunity to identify small molecules that preferentially regulate one pathway or the other.

The critical role for LXRα in regulating hepatic fatty acid synthesis [19, 46, 66, 81] raises the possibility that small molecule LXR antagonists may have clinical benefits for patients with NAFLD [114, 115]. The incidence of NAFLD is rising in concert with the obesity epidemic, however, the etiology of this disease is complicated. The transition from simple fatty liver to NASH with its associated inflammation and fibrosis appears to be what puts patients at increased risk for cirrhosis, liver failure, and liver cancer [113]. Since elevated hepatic cholesterol levels have been linked to the incidence and severity of NASH studies [116–119] using LXR antagonists should proceed cautiously. LXR antagonists may be useful relatively early during the disease course to reduce fatty liver before patients transition to NASH but could prove detrimental at later stages. Continued study of the role of LXR and cholesterol sensing in hepatocytes, Kupffer cells, infiltrating immune cells, and hepatic stellate cells will be needed to maximize the potential therapeutic activity of LXR ligands for the treatment of liver disease.

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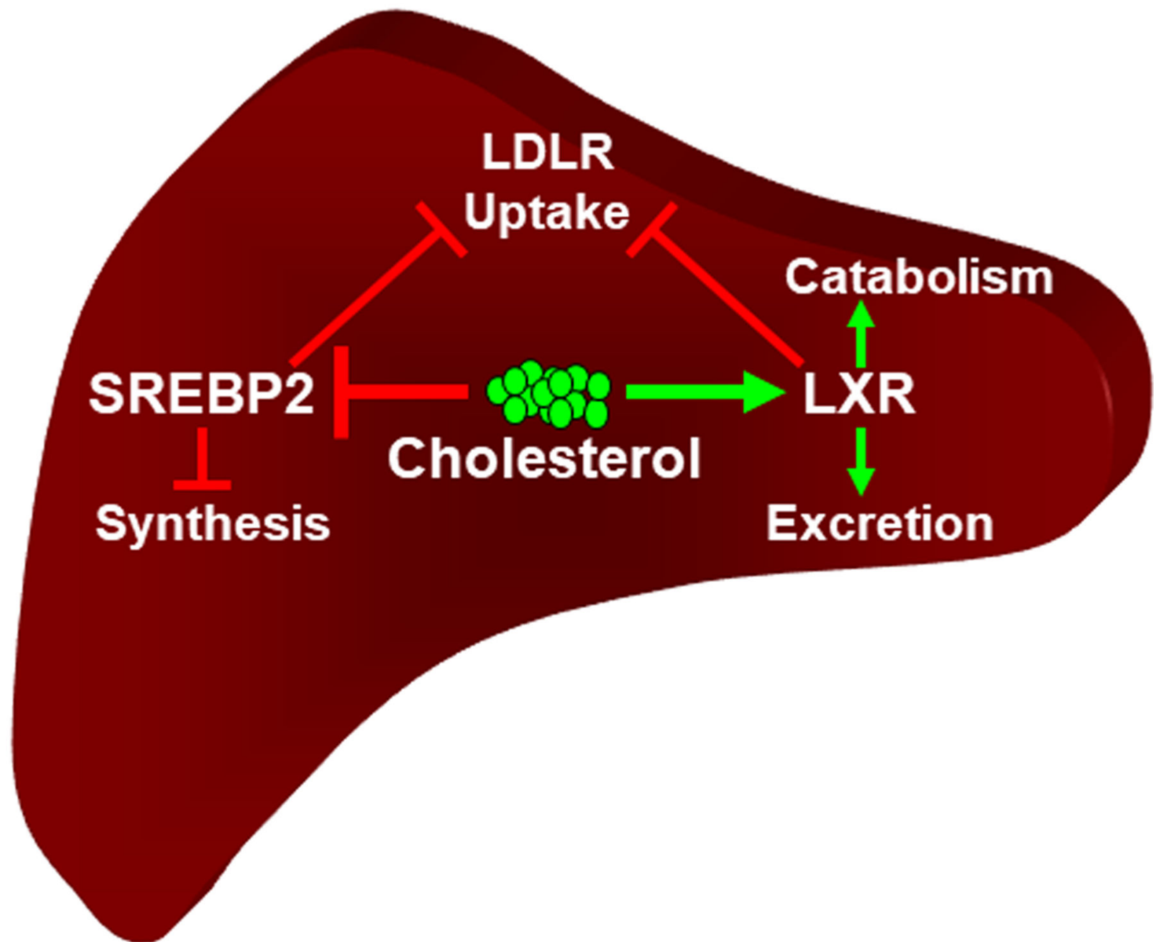

Figure 1. Cholesterol sensing in the liver.

Figure illustrates the response to elevated cholesterol levels. LXRα mediates a feed forward pathway that increases cholesterol catabolism to bile acid and cholesterol excretion while decreasing cholesterol uptake via expression of the LDL receptor. Negative feedback control is mediated by cholesterol dependent inhibition of the transcriptional activity of SREBP2 leading to decreases in cholesterol synthesis and cholesterol uptake. Green arrows indicate positive activation. Red bars indicate inhibition. See the text for details.

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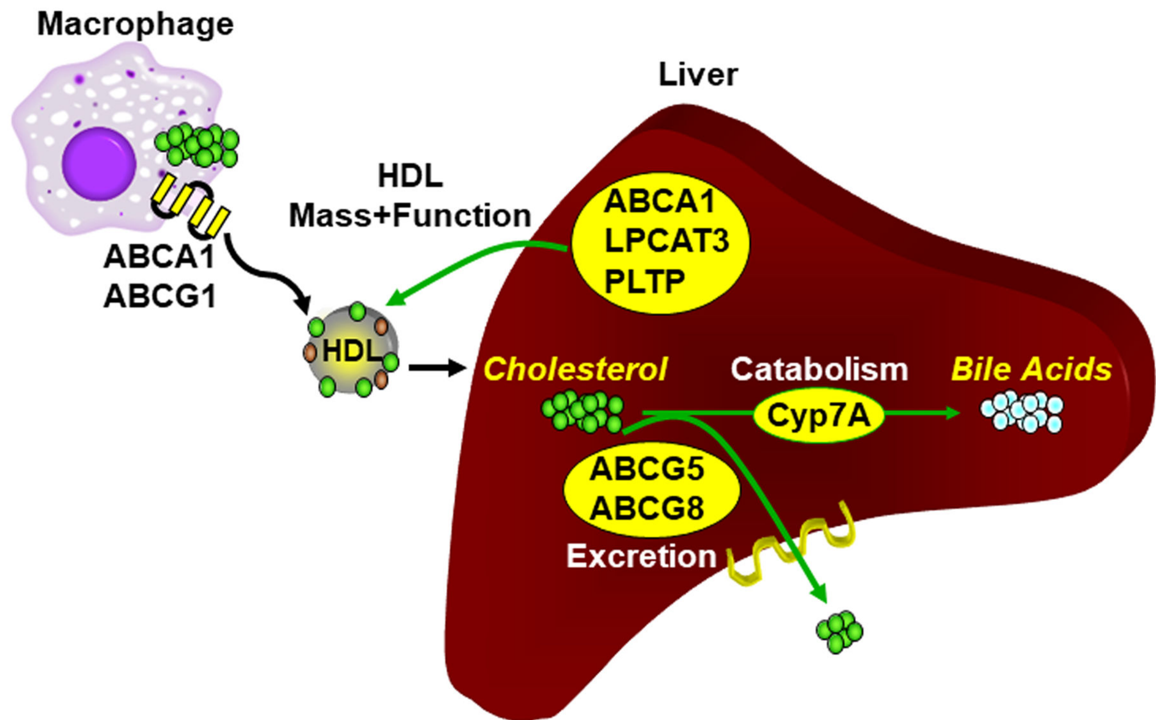

Figure 2. Hepatic LXR controls RCT.

In the liver LXRα controls the flux through the RCT pathway by regulating cholesterol catabolism to bile acids and cholesterol excretion. Hepatic LXRα also controls the ability of HDL to accept cholesterol from macrophages by controlling the number of HDL particles and the functional activity HDL on a per particle basis. See the text for details.

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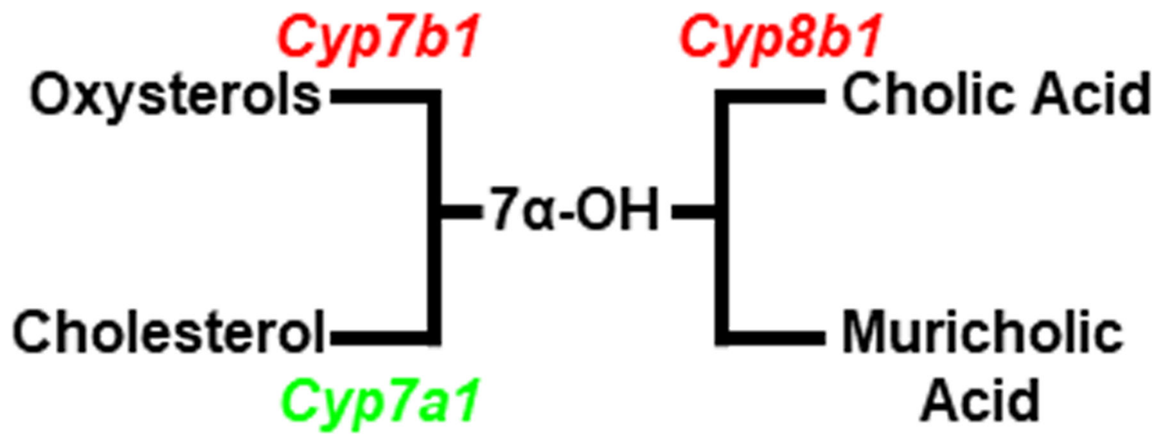

Figure 3. Control of bile acid synthesis by LXR.

By increasing expression of *Cyp7a1* and decreasing expression of *Cyp7b1* LXR activation drives the catabolism of cholesterol to bile acids while preserving oxysterol levels. LXR dependent repression of *Cyp8b1* drives the composition of bile acids to muricholic acid which decreases the intestinal absorption of cholesterol.

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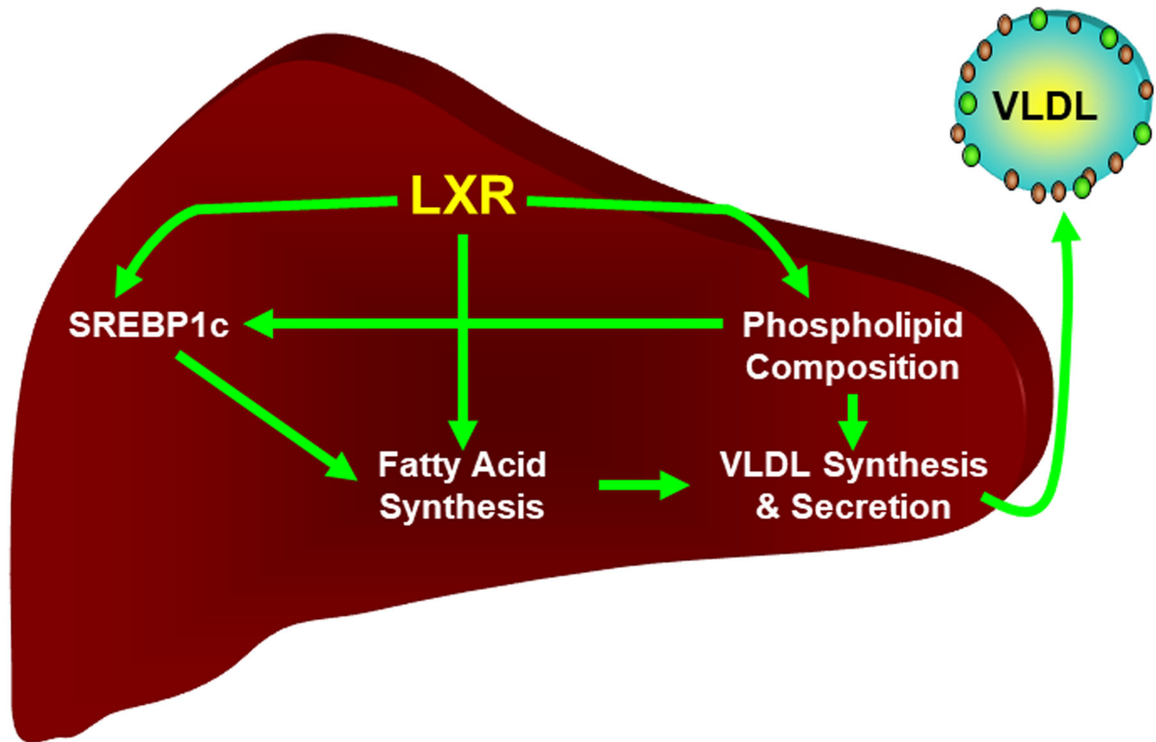

Figure 4. Control of fatty acid synthesis and VLDL secretion by LXR.

LXR directly regulates the expression of genes encoding enzymes required for fatty acid synthesis as well the gene encoding SREBP1c, a second lipogenic transcription factor. Regulation of phospholipid composition indirectly influences the lipidation and secretion of VLDL and the activity of SREBP1c. See the text for details.

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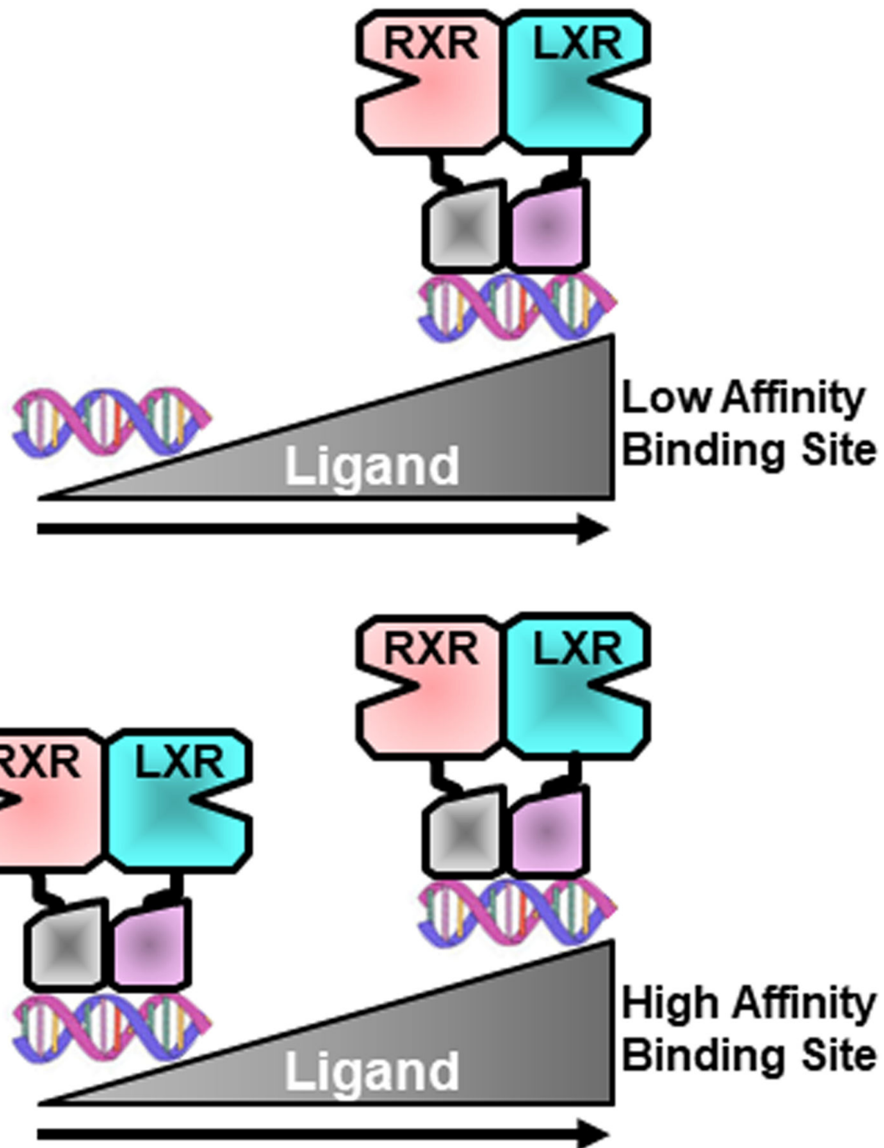

Figure 5. Model for the differential regulation of LXR target genes in hepatocytes.
We suggest that genes with relatively low affinity LXR binding sites (top) are only regulated by LXR when ligand concentrations are high such as after feeding with high cholesterol diets or after treatment with potent synthetic ligands. Genes with relatively high affinity binding sites ae regulated by LXRs even when ligand concentrations are relatively low. See text for details.